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13. ABSTRACT ***(Maximum 200 Words)***

The gene of the nuclear receptor coactivator AIB1 is amplified in breast cancer cell lines as well as in breast tumor tissue. AIB1 mRNA is often highly expressed in primary breast tumors and is has been shown that AIB1 enhances estrogen and progesterone dependent transcription *in vitro*. Therefore it has been postulated that AIB1 contributes to the development of breast cancer. However, it is currently not known what the precise role of AIB1 is in the development of breast cancer. Our strategy to address this question is to establish breast cancer cell lines in which we can regulate AIB1 levels with ribozymes in order to determine the impact of reduced AIB1 gene expression on the phenotype and angiogenic or invasive properties of breast cancer cells. During the first year we developed MCF-7 breast cancer cells in which we are able to downregulate AIB1 protein levels by up to 90%. Furthermore, downregulation of AIB1 levels reduced the ability of the progesterone receptor to fully activate a progesterone regulated test promoter. We anticipate that our study will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.

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Table of Contents

Introduction

Small fat soluble hormones such as steroids, retinoids and vitamin D_3 play pivotal roles in the control of breast cancer proliferation and differentiation. The biological effects of these molecules are mediated through intracellular receptor proteins. Estrogens stimulate proliferation of estrogen receptor (ER) positive breast cancer cells and the ER status of a breast tumor is predictive of the outcome of the disease. Therapy (such as tamoxifen) targeted at reducing the estrogenic stimulus to the breast has been shown to be effective. Conversely retinoids and vitamin D_3 are strongly growth inhibitory in breast tumor cells and analogues of these compounds are currently being tested for efficacy in breast cancer. Recently the discovery of proteins known as steroid receptor co-activators has led to another level of complexity to our understanding of how hormones exert their effects[1,2]. Of particular interest is the co-activator **AIB1 (amplified in breast cancer 1)**, which interacts with the estrogen receptor (ER) [3]. Interestingly, the AIB1 gene was found to be amplified and AIB1 expression is increased in breast tumors and breast cancer cell lines [3,4]. **However, it is currently not known what the role of AIB1 amplification is in the development of breast cancer nor is it known whether detection of AIB1 overexpression will be valuable for diagnosis of breast cancer or for prognosis of disease outcome.** The possible role of AIB1 in breast cancer is further complicated by studies which demonstrate that AIB1 can also interact with retinoid, thyroid, Vit D_3, PPAR and androgen receptors [5-7]. **This broad ability to potentiate the effects of a number of hormone receptors leads to the question of what is the precise role of the AIB1 amplification in breast cancer?**

In this proposal we will investigate the hypothesis that overexpression of AIB1 in breast cancer is important for breast tumor development by impacting upon nuclear hormone receptor function. In particular we wish to investigate if AIB1 is required for expression of a gene(s) critical for breast cancer development. The novel approach we will take to study this question will be to develop hammerhead ribozymes to target and cleave AIB1 thus producing a selective reduction of this coactivator in breast cancer cells. Ribozymes are molecules of RNA which can cleave a specific target RNA (in this case AIB1) and thus selectively reduce expression of the AIB1 protein in the cell. In addition, the potential development of ribozymes as potent therapeutic agents [8] makes the translation of these results into possible therapies realistic. **In this study we will examine the idea that the nuclear receptor coactivator AIB1 is rate-limiting for breast cancer development and that selective targeting of this coactivator will be useful for future development of novel therapies leading to reduced proliferation or metastatic potential of these cells.** Our first experiments will focus on designing ribozymes which selectively decrease AIB1 *in vivo*. Using these reagents as tools, we will then determine the impact of reduced AIB1 gene expression on estrogen, progesterone, retinoid, Vit D_3, PPAR and AR receptor function in breast cancer cell lines. Finally we will determine if reduction of AIB1 mRNA can influence the progression of breast cancer *in vivo*. **We anticipate that these experiments will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.**

Proposal Body

In the approved Statement of Work three Tasks were outlined.

Task 1: Development of AIB1 selective targeted ribozymes (month 1-12)

Task 2: Determining the impact of reduction in AIB1 mRNA on the phenotype of breast cancer cells (6-30 month)

Task 3: To determine if the reduction in AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines (12-36 month)

The following report about our progress includes data showing progress concerning Task 1 and Task 2. Specifically:

Task 1: i) The first goal of our studies was the development of a series of plasmids that will specifically target AIB1 mRNA in vectors with the CMV promoter and also in vectors with the regulatable tetracycline promoter.

Design of ribozymes targeted to cleave AIB1 mRNA

Ribozymes are RNA molecules which have catalytic activity enabling them to cleave target RNA (here AIB1-mRNA) which is then rapidly degraded in the cell. We developed five ribozymes for this project based on successful ribozyme strategies we have used previously [9]. The ribozymes contain the hammerhead-ribozyme core sequence flanked by 10 sequence specific bases, directed against AIB1, on either site of the core structure. Four ribozymes are directed specifically against different regions of the coding region of AIB1 and one ribozyme is directed against the 3' untranslated region of AIB1 (see Fig. 1 in Appendix). The sites of cleavage were chosen because of little homology with other known members of the nuclear coactivator family and did not have significant homology to any other known mRNA in the human gene database. The selection of the ribozymes was based on sequence homology searches by using the University of Wisconsin Genetics Computer Group (GCG) Program package and the Basic Local Alignment Search Tool (BLAST) Program for nucleic acids from the National Center for Biotechnological Information (Bethesda, MD).

The ribozyme expression plasmids were made by synthesizing complimentary oligonucleotides harboring the ribozyme plus the flanking complementary sequences to the AIB1 target mRNA. After annealing, the oligonucleotides were inserted into i) a vector, pRcCMV, which harbors a multiple cloning site down-stream of the CMV promoter and ii) a vector, p605, which harbors a pTET operon driving the expression of the ribozyme (tet-regulation). The oligonucleotides were synthesized with overhangs to restriction sites which allowed directional cloning. We have successfully used this methodology for the expression (constitutive or under tetracyclin (tet) regulation) of ribozymes in a number of different cell types [9-11]. The CMV vector harbors a neomycin resistance gene which enables us to select cells which carry the AIB1 expression vector

as a stable insert. (The tet-regulated AIB1 expression vectors were cotransfected with a vector containing resistance against purimycin). All plasmids were sequenced before we proceeded with transfections of the different cell lines.

i) The second goal of Task 1 was to stably transfect MCF-7 and T47-D cells with these vectors.

a) Stable transfection of the plasmids which contain constitutive expressing AIB1 ribozymes

We transfected four of the five plasmids which constitutive express the AIB1 ribozymes (Rz-12, Rz-15, Rz-29 and Rz-31) into the two breast cancer cell lines MCF-7 and T-47D, which are high and low AIB1 mRNA expressing lines respectively [3]. We prefered to test the activity of ribozymes in stable cell lines because it is difficult to see reductions in target RNA in transient assays where only a portion of the cells will receive the ribozyme vector but all cells express AIB1 mRNA. After stable selection with G418 we determined whether the endogenous AIB1 mRNA levels were reduced in mass stable transfected cell lines by Northern blot analysis of ribozyme-transfected cells versus cells (wt) which received the empty expression vector (pRcCMV) (see below).

b) Stable transfection of the plasmids which contain tetracyclin regulated AIB1 ribozymes

A major potential obstacle in achieving a constitutive ribozyme mediated AIB1 depletion in stably transfected cells is that AIB1 expression may confer a growth advantage, thus making selection of low AIB1 expressing cells difficult. To circumvent this potential problem we transfected the hammerhead ribozymes under the control of a tetracycline-regulated promoter to evaluate the effect of conditional AIB1 depletion *in vitro* and *in vivo* on breast cancer growth. To achieve this, we used a MCF-7 TA-expressing cell line which has been transfected with a tetracycline transactivator/VP16 fusion protein vector (pUHG15-1) and was already available in the laboratory [12]. A high expressing clone of these cells was then used for co-transfection of the pTET operon driving ribozyme expression vectors and the purimycin resistence vector. The resulting cell lines did harbor TET expression vectors which drive expression of the ribozyme when tetracycline (doxycycline) is removed from the media [12]. We have used this system before and found it to be effective in both breast and ovarian cancer cell lines [12].

i) Final goals of Task 1 were to select clonal lines with constitutive or regulatable levels of AIB1 mRNA expression and tio characterize the effect of the AIB1 ribozymes using Northern blot analysis of AIB1 expressing cells.

Isolation and characterization of stably transfected MCF-7 and T47-D cells

Some preliminary results of the Northern blot analysis from MCF-7 cells have been included in the original proposal (see Fig. 2). Basically, what we observed was a 20-40% reduction of AIB1 mRNA levels in MCF-7 with Rz-12 and Rz-29. In contrast to these promising results in MCF-7 cells, we did not see a significant reduction of AIB1 levels in T47D cells by Northern blot analysis. Based on these data and the results that we obtained by stable transfection of the tet-regulated plasmids (see below), we decided to focus our work on the cell lines containing tet-regulated AIB1 ribozymes.

After transfection of the MCF-7 cells with the tet-regulated AIB1 ribozymes, we immediately isolated clonal cell lines which were then tested for AIB1 ribozyme expression by Northern and Western blot analysis. AIB1 mRNA and protein levels were tested by comparison of AIB1 levels in cells treated with doxocyclin, which has the same function as tetracycline, (inhibition of ribozyme) or left untreated (active ribozyme). We observed a reduction of AIB1 levels in several clones, with the highest reduction in MCF-7 clones transfected with Rz-12 and Rz-23. AIB1 mRNA levels were reduced between 12 and 40% for clone 12-9 and 23-9 respectively (Fig. 2A). In Western blot analysis we obtained an even stronger reduction of AIB1 levels. In MCF-7 cells transfected with Rz 12 (clone 12-9) we observed a reduction of AIB1 protein levels of 40% and in cells with Rz-23 (clone 23-9) we obtained a reduction of AIB1 levels of up to 90% (Fig. 2B).

In conclusion, we were able to design, construct and transfect several plasmids containing AIB1 ribozymes and to obtain stably transfected MCF-7 cell lines in which AIB1 protein levels were reduced by up to 90% compared to wild type cells.

Task 2: The goals of our studies regarding Task 2 were i) to determine the impact of reduction of AIB1 mRNA on gross phenotype changes in cells including proliferation, differentiation and apoptosis and ii) to determine the effect of AIB1 reductions on gene expession and promoter activity of individual hormones.

Currently we are performing proliferation and soft agar assays with MCF-7 clone 12-9 and clone 23-9 (both express tet-regulated ribozymes). We are testing whether the growth of these cells is differently regulated by various hormones (e.g. estrogens, progesterone, androgens) with regard to AIB1 levels.

In addition, we test whether AIB1 levels influence the inducibility of estrogen and progesterone responsive promoters in MCF-7 cells. Preliminary data show a reduction of progesterone responsiveness of a test promoter after reduction of AIB1 levels Fig. 3.

Task 3: The goals of our studies regarding Task 3 were to determine if the reduction in AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines.

These goals will be, as planned, performed in years two and three of the funding period.

Key Research Accomplishments

- We designed, constructed and sequenced i) four plasmids which constitutive express AIB1 and ii) five plasmids which express the AIB1 ribozymes in a regulated fashion under the control of the tet-off system (regulatable ribozymes).

- We generated stable MCF-7 and T47-D cell lines which i) express constitutive AIB1 ribozymes and ii) which express tetracyclin regulated AIB1 ribozymes (only MCF-7).

- We identified and isolated mass and clonal MCF-7 cell lines which had reduced AIB1 mRNA levels of up to 40% compared to control cells and reduced AIB1 protein levels of up to 90%.

- We could demonstrate that a a test promoter was less inducible by progesterone in MCF-7 cells which had reduced AIB1 levels.

Reportable Outcomes

- Establishment of MCF-7 clonal cell lines which express constitutive active or regulatable ribozymes directed against AIB1.

- List, H.-J., Lauritsen, K., Reiter, R., Wellstein, A. and Riegel, A.T. The nuclear hormone receptor coactivator AIB1 is essential for estrogen and progesterone mediated activity in MCF-7 breast cancer cells. Manuscript in preparation.

Conclusions

In this study we want to examine whether the nuclear receptor coactivator AIB1 is rate-limiting for breast cancer development and whether selective targeting of this coactivator will be useful for the future development of novel therapies leading to reduced proliferation or metastatic potential of these cells. Specifically, we want to answer the question of "what is the precise role of AIB1for breast cancer development *in vivo* considering its broad ability to potentiate the effects of a number of hormone receptors *in vitro*"?
During the first year of this project we have established an exellent tool to answer these questions. We developed MCF-7 breast cancer cell lines in which we are able to regulate AIB1 protein levels by targeting AIB1 with regulatable ribozymes specifically directed against AIB1 mRNA. Preliminary results indicate that downregulation of AIB1 levels reduce the ability of the progesterone receptor to fully activate a progesterone regulated target promoter.We anticipate that the proposed experiments, outlined in the approved Statement of Work, will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.

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Appendix

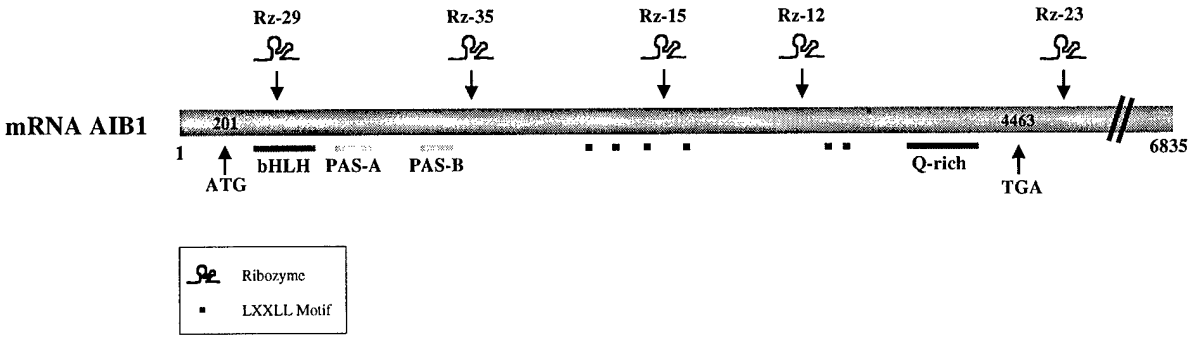

Fig.1: Diagram of the structure of the mRNA of AIB1.The positions of the ribozymes used in this study are indicated by the arrows.

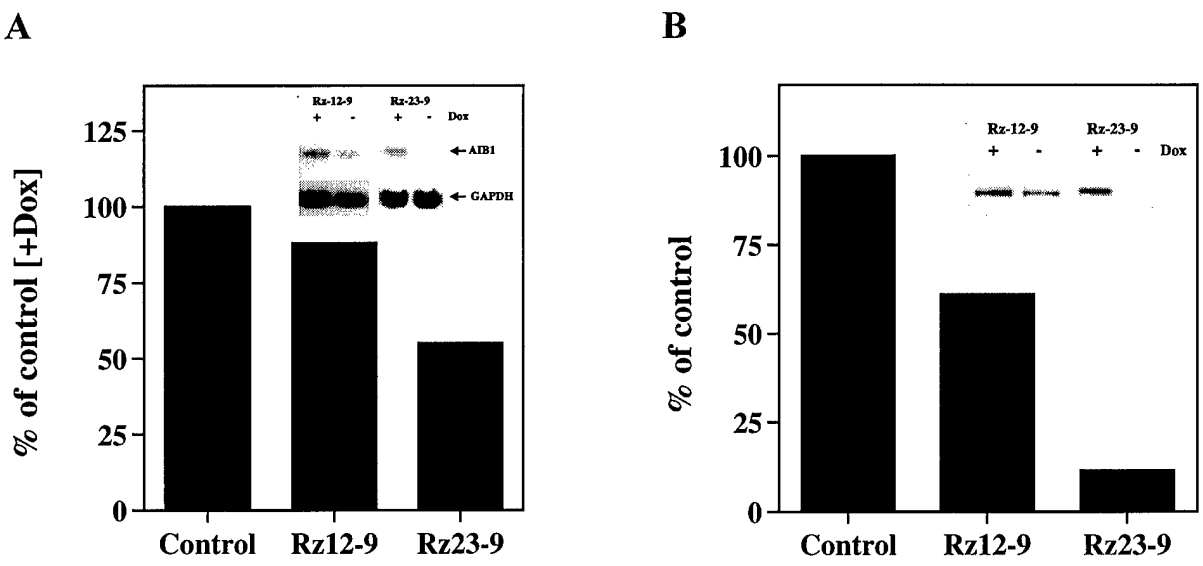

Fig.2: Reduction of AIB1 levels in MCF-7 cells. (A) Northern blot analysis of MCF-7 cells stably transfected with tetracyclin regulated ribozyme Rz12 (clone 12-9) and Rz-23 (clone 23-9). AIB1 levels were corrected for GAPDH expression. (B) Western blot analysis of MCF-7 cells stably transfected with tetracyclin regulated ribozyme Rz12 (clone 12-9) and Rz-23 (clone 23-9).

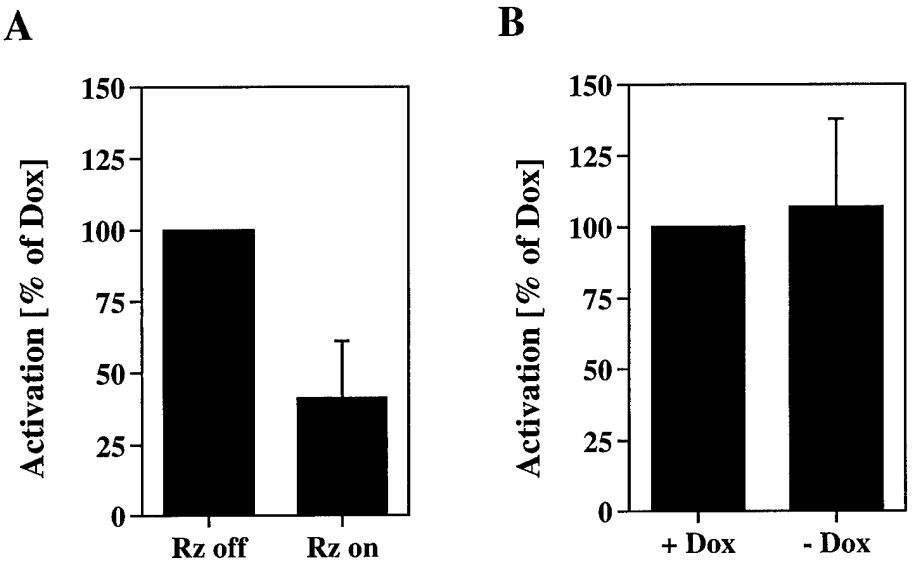

Fig.3: Influence of AIB1 levels on the activation of a progesterone responsive promoter (pMMTV-GRE). (A) Activation of pMMTV-GRE by R5020 in MCF-7 breast cancer cells (clone 23-9) transfected with the regulatable AIB1 ribozyme Rz-23 (see Fig.1). Cells were treated with doxycycline (Rz off: normal AIB1 level) or left untreated (Rz on: low AIB1 level). (B)Activation of pMMTV-GRE by R5020 in wild type MCF-7 breast cancer cells. Cells were treated with doxycycline or left untreated